Optimization of a Versatile in Vitro Transcription Assay for the Expression of Multiple Start Site TATA-less Promoters†

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ABSTRACT: Previous work from our laboratory has allowed for the subdivision of RNA polymerase II TATA-less promoters into two classes: those that initiate at a single start site (SSS) and those that initiate at multiple start sites (MSS). MSS promoters are defined by the lack of a TATA box and the presence of a transcription initiation window and a downstream MED-1 element (GCTCCC/G) [Ince, T. A., and Scotto, K. W. (1995) *J. Biol. Chem. 270*, 30249−30252]. Further insight into the mechanisms regulating TATA-less MSS promoters has been hampered by the lack of an in vitro transcription assay in which multiple start sites can be reproduced. In the present study, we describe the development of a versatile in vitro transcription system optimized for the expression of MSS promoters, termed the multiple promoter comparison (MPC) assay. By alteration of assay parameters including template length, cation and nucleotide concentrations, and RNA isolation method, the accurate and robust transcription of two MSS promoters, pgp1 (hamster P-glycoprotein class I homologue) and HPRT (human hypoxanthine phosphoribosyltransferase), was accomplished. Moreover, both TATA-containing and TATA-less single start site promoters were also transcribed in the MPC assay, making this the first general in vitro transcription system for the simultaneous analysis of all three classes of RNA polymerase II genes.

The past two decades have witnessed an explosion in our understanding of the complex interplay among core DNA elements and the protein factors that regulate basal RNA polymerase II (Pol II)[1] mediated transcription. Much of this knowledge comes from analyses of the transcription of TATA-containing promoters in cell-free systems (*1*). Located 25−30 base pairs upstream of the transcription start site, the TATA box is the site of nucleation of the Pol II preinitiation complex (PIC), which includes the general transcription factors (GTFs) TFIIA, -IIB, -IID, -IIE, -IIF, and -IIH as well as Pol II. The PIC is recruited to TATA-containing promoters through the direct binding of TBP (TATA-binding protein), a component of TFIID, to the TATA element. The interaction of the PIC with the promoter is then stabilized through both protein−protein and protein−DNA interactions (for review, see refs *2*−*6*).

The early notion that all promoters required a TATA box in order for transcription to occur was refuted by the finding that more than half of all known promoters lack such an element (*7*). This resulted in the grouping of promoters into two classes, TATA-containing and TATA-less. A portion of TATA-less promoters direct transcription from a single start site or a narrow cluster of start sites surrounding the major +1 site; many of these promoters have been shown to contain an alternative core element, the initiator (Inr), which encompasses the transcription start site and appears to be involved in nucleation of the PIC in the absence of a TATA motif (*8*−*10*). Although the mechanism by which the Pol II PIC interacts with promoters lacking a TATA box has not been fully elucidated, several DNA-binding proteins, including TFII-I (*11*−*14*) and YY1 (*13*, *15*), have been shown to interact with the Inrs of certain genes, suggesting roles in positioning the PIC on these promoters. Intriguingly, a TFIID complex containing a TATA-binding-defective TBP is capable of directing accurate transcription from TATA-less promoters (*16*), indicating that the interaction of TBP with this promoter class is fundamentally different than its interaction with TATA-containing promoters.

Recent studies in our laboratory have allowed for the further subdivision of TATA-less promoters into two

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[1] Abbreviations: AdML, adenovirus 2 major late; bp, base pair; β Pol, DNA polymerase β; GTFs, general transcription factors; HPRT, human hypoxanthine phosphoribosyltransferase; iCCAAT, inverted CCAAT box; Inr, initiator; MDR1, multidrug resistance, human class I homologue of P-glycoprotein; MPC, multiple promoter comparison; MSS, multiple start site; pgp1, hamster P-glycoprotein (class I homologue); PIC, preinitiation complex; Pol II, RNA polymerase II; SSS, single start site; SV40, simian virus 40; TBP, TATA-binding protein; WT, wild type.

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classes: those that utilize a single start site (SSS) and those that initiate at multiple start sites (MSS). It is notable that some of these start sites in MSS promoters are embedded in initiator sequences, while many others are not, suggesting that additional elements/factors participate in MSS selection/utilization. Apropos of this, we have shown that the MSS initiation window within the hamster P-glycoprotein (pgp1) promoter can be differentially utilized in different cell types. In Chinese hamster lung cells, transcription of the endogenous pgp1 promoter begins at a single site (+1) in drug-sensitive parental cells while transcription in multidrug-resistant subclones initiates at +1 as well as at additional downstream sites (+32, +42, +52, +67) (*17*). Utilization of the downstream start sites in vivo is mediated, at least in part, by a novel downstream element designated MED-1 (GCTCCC/G) (*18*), which we have identified in the majority of MSS promoters to date (*19*). The factors/elements regulating +1 selection remain elusive.

Aside from the identification of MED-1, little is known regarding the regulation of start site selection in MSS TATA-less promoters, either in vivo or in vitro. It is clear from the literature that one of the major reasons for the lack of studies focused on the transcriptional mechanism of MSS promoters has been the difficulty encountered in accurately reproducing start sites in vitro. Although in vitro transcription of TATA-containing promoters is fairly routine, to our knowledge there is only a single report in which TATA-less MSS transcription has been recapitulated (*20*); unfortunately, this approach was not successful in our hands when other MSS promoters were used as templates (Lin, Ince, and Scotto, unpublished results).

MSS promoters as a class are inherently difficult to analyze in vitro for several reasons: (1) Generally, they are extremely weak when compared to TATA-containing promoters; often the transcription of these promoters is below the level of detection using current methods. This difficulty in detection is further exacerbated by the splitting of signals over multiple transcripts. (2) MSS promoters are generally GC rich, making detection of endogenous start sites with primer extension and nuclease protection assays both difficult and subject to considerable artifacts. (3) Since the transcription initiation window of MSS promoters includes many G residues, as well as the G-rich MED-1 element that participates in start site utilization in vivo, they are not amenable to study using the classic G-less cassette assay, which is the most sensitive and robust transcription assay. Thus, a novel and versatile system for the evaluation of MSS promoters is critical to the dissection of their complex regulation. In the present report, we describe the development of an in vitro transcription assay that has been optimized for expression of MSS promoters using pgp1 as a model promoter and discuss the usefulness of this assay system for all three Pol II promoter classes.

MATERIALS AND METHODS

Cell Lines, Nuclear Extracts, and Transfections. HeLa S3 cells were grown in Joklik's MEM media with 5% fetal calf serum, 2.0 g/L $NaHCO_3$, and 1% penicillin/streptomycin at the National Cell Culture Center (Minneapolis, MN), harvested at a cell density of 1×10^6 cells/mL by centrifugation for 10 min at 2500*g*, and washed twice in PBS (Ca^{2+}/Mg^{2+} free). The cell pellets were shipped overnight on wet ice for the preparation of nuclear extract. Extracts were prepared according to Dignam (*21*, *32*) and used at a concentration of 8−10 mg/mL. Human KB-3-1 epidermoid carcinoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. KB-3-1 cells were transfected with 0.5 μg of wild-type or mutant MDR1LB luciferase reporter constructs using lipofectin (Invitrogen, Rockville, MD) following the vendor's recommendations. The total amount of DNA was adjusted to 2.0 μg/well by the addition of sonicated salmon sperm DNA (Stratagene, La Jolla, CA). Transfected cells were incubated for 40 h before harvesting, and luciferase assays were performed as recommended by the vendor (Promega, Madison, WI). The luciferase activity was normalized against protein concentration as determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Plasmids. The plasmid containing the pgp1 promoter, pgpLucB, has been previously described (*19*). The plasmid containing the −1202/+118 fragment of the MDR1 promoter, MDR1LB, was constructed by cloning the *Xma*I/*Sty*I fragment of the promoter into the *Xma*I/*Nhe*I sites of pGL2B (Promega). The plasmids containing the inverted CCAAT box mutations of the MDR1 promoter, M1 and M2, were described previously (*27*). The plasmid containing the human HPRT promoter, HPRTLBS, included a 467 bp promoter fragment amplified from HeLa genomic DNA using the oligonucleotides 5′-ACGCGATGACTGGAACCCG-3′ and 5′-AGCTGCTCACCACGACGCC-3′ and cloned into the *Kpn*I/*Sac*I site of pGL2B. PAdβ, which contains the AdML promoter (*22*), WWP-LucB, which includes the human $p21^{WAF1/CIP1}$ promoter (*23*), and pBP8, which contains the DNA polymerase β promoter (*24*), were described previously. pGL2P (SV40 promoter) was purchased from Promega.

Preparation of DNA Templates. DNA templates were prepared by PCR amplification of appropriate plasmids, using the oligonucleotides described below and Taq polymerase (Invitrogen). Resulting promoter fragments were 400−500 bp in length. DNA template (0.05−0.9 pmol) was used for each reaction, as described in the figure legends.

The oligonucleotides used for preparation of PCR templates for in vitro transcription were as follows: for pgpLucB, template A, 5′-TGGCGGGGATGTGAGTTCATCAAC-3′ and 5′-CTTTATGTTTTTGGCGTCTTCC-3′; for pgpLucB, template B, 5′-TGGCGGGGATGTGAGTTCATCAAC-3′ and 5′-TCCTCTAGAGGATAGAATGGCGCCGG-3′; for MDR1LB, 5′-TCTAGAGAGGTGCAACGGAAGCCAG-3′ and 5′-CTTTATGTTTTTGGCGTCTTCC-3′; for PAdβ, 5′-AGAAACTCGGACCACTCTGAGACGAAGGCT-3′ and 5′-CTCAGGTCCCTCGGTGGCGGAGT-3′; for WWP-LucB, 5′-GTCTATTTGAAATGCCTGAAAGCAGAGGGG-3′ and 5′-CTTTATGTTTTTGGCGTCTTCC-3′; for pGL2P, 5′-TGTATCTTATGGTACTGTAACTG-3′ and 5′-CTTTATGTTTTTGGCGTCTTCC-3′; for pBP8, 5′-CAGTCACGACGTTGTAAAACGACGGC-3′ and 5′-GGGCAAGAATGTGAATAAAGGCCGG-3′; for HPRTLBS, 5′-AGGGAGCCCTCTGAATAGGAGACTGAGT-3′ and 5′-CTTTATGTTTTTGGCGTCTTCC-3′.

In Vitro Transcription. In vitro transcriptions were performed under either standard assay conditions, multiple promoter comparison (MPC) conditions, or MPC conditions

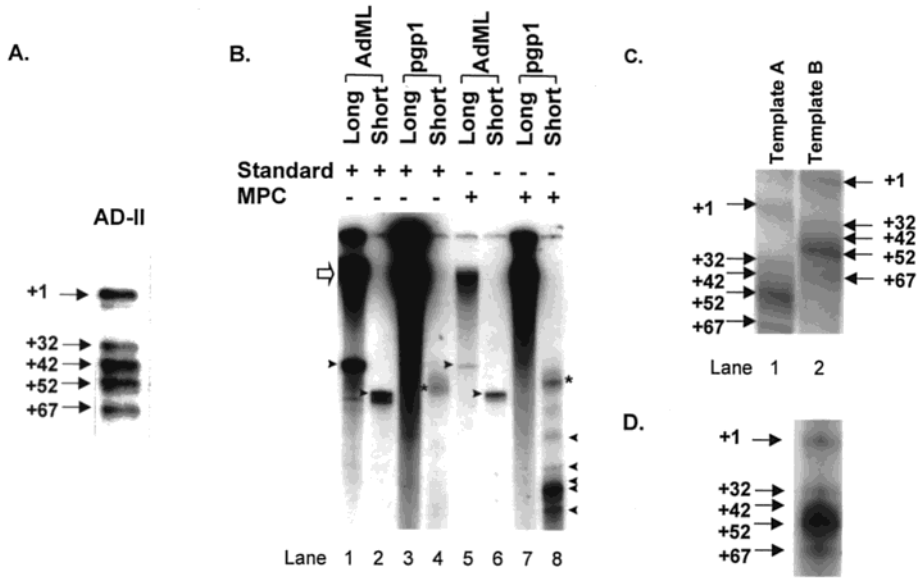

FIGURE 1: Multiple start site utilization is optimal under MPC conditions. (A) RNase protection analysis of pgp1 RNA from drug-resistant DC-3F/AD-II cells, taken from ref *17*. (B) In vitro transcription of the AdML and pgp1 promoters using both standard and MPC conditions with either linearized plasmid (long) or PCR (short) templates. AdML was transcribed under standard (lanes 1 and 2) or MPC (lanes 5 and 6) conditions. The pgp1 promoter was transcribed under standard (lanes 3 and 4) or MPC (lanes 7 and 8) conditions. Either long (lanes 1, 3, 5, and 7) or short (lanes 2, 4, 6, and 8) promoter-containing fragments were used as templates. Black arrowheads designate start sites. The empty arrow designates nonspecific transcription from the plasmid template. Asterisks indicate nonspecific transcription from the short linear template of the pgp1 promoter. The molar concentration of the DNA templates (long and short) for each promoter was kept constant under all conditions. The concentration of the pgp1 templates is 10-fold higher than the concentration of the AdML templates. (C) In vitro transcription of the pgp1 promoter in the MPC assay using PCR templates with different 3′ lengths but the same 5′ ends. Template B is 30 bp longer than template A. (D) A longer gel with higher resolution showing the five start sites of the pgp1 promoter transcribed in vitro using MPC conditions.

with variations as described in Results (semi-MPC conditions).

(A) Standard (S) Conditions. In vitro transcription under standard conditions was performed as described (*1*). HeLa nuclear extract and DNA templates were preincubated in a 25 μL reaction buffer containing 10 mM HEPES, pH 7.9, 10% glycerol, 60 mM KCl, 7.5 mM $MgCl_2$, 0.5 mM DTT, and 0.8 unit/mL RNaseOUT (Invitrogen) at 30 °C for 45 min, followed by the addition of 25.5 μM [α-^{32}P]GTP (12.8 Ci/mmol), 600 μM ATP, 600 μM UTP, and 600 μM CTP. Incubation was continued for an additional 45 min at 30 °C. The reaction was stopped by the addition of 75 μL of stop solution containing 1% SDS, 100 mM sodium acetate, pH 5.5, and 1 mg/mL tRNA, extracted with phenol/chloroform, and precipitated by ethanol.

(B) Multiple Promoter Comparison (MPC) Conditions. In vitro transcriptions were performed by preincubating HeLa nuclear extract and DNA templates in a 25 μL reaction buffer containing 30 mM HEPES, pH 7.9, 20% glycerol, 70 mM KCl, 2 mM $MgCl_2$, 4 mM DTT, 1% DMSO, 2% PEG-8000, and 0.8 unit/mL RNaseOUT at 27 °C for 45 min, followed by the addition of 3.5 μM [α-^{32}P]GTP (114 Ci/mmol), 400 μM ATP, 400 μM UTP, and 400 μM CTP. Incubation was continued for an additional 45 min at 27 °C. The reaction was stopped, and RNA was precipitated by the addition of 275 μL of solution D (4 M guanidinium thiocyanate, 2.5 mM sodium citrate, pH 7.0, 0.5% sarkosyl, 0.1 M 2-mercaptoethanol), 10 μg of yeast tRNA, 300 μL of 2-propanol, and 1 μL of pellet paint coprecipitant (Novagen, WI). RNAs were separated by electrophoresis in a 6% urea−polyacrylamide gel and autoradiographed.

The experiments shown in Figures 2 and 3 were performed under semi-MPC conditions where only Mg^{2+} concentration (Figure 2A), nucleotide concentration (Figure 2B), KCl concentration (Figure 3B), preincubation time (Figure 3C), or reaction time (Figure 3D) were varied from MPC conditions as described in the figure legends.

RESULTS

Development of the MPC Assay: In Vitro Transcription under Standard versus MPC Assay Conditions. The pgp1 promoter utilizes multiple start sites in drug-resistant Chinese hamster DC-3F/AD-II cells in vivo (Figure 1A). Initially, we attempted to recapitulate these start sites in vitro under the assay conditions routinely used for the analysis of single start site promoters, using either supercoiled or linear pgp1 promoter templates. However, although transcription of the TATA-containing SSS adenovirus 2 major late (AdML) promoter was readily detected using supercoiled plasmid templates (data not shown) or a linearized plasmid template (Figure 1B, lane 1), no specific pgp1 transcripts were observed under these conditions (Figure 1B, lane 3) (empty arrow denotes nonspecific transcription from the plasmid template). This led us to optimize an assay system specifically suited for the analysis of TATA-less MSS promoters. By varying a number of assay parameters that will be discussed in more detail below, a system which we term the multiple promoter comparison (MPC) assay was developed. Transcription of both the AdML and pgp1 promoters using the MPC conditions is shown in Figure 1B, lanes 5−8. Notably, while transcription from the AdML promoter was favored under standard conditions using long (lane 1) or short (lane 2) linear templates, accurate transcription of the multiple pgp1 transcripts was only observed using the MPC assay conditions and a short (~430 bp) linear template (compare lanes 3, 4, and 7 with lane 8).

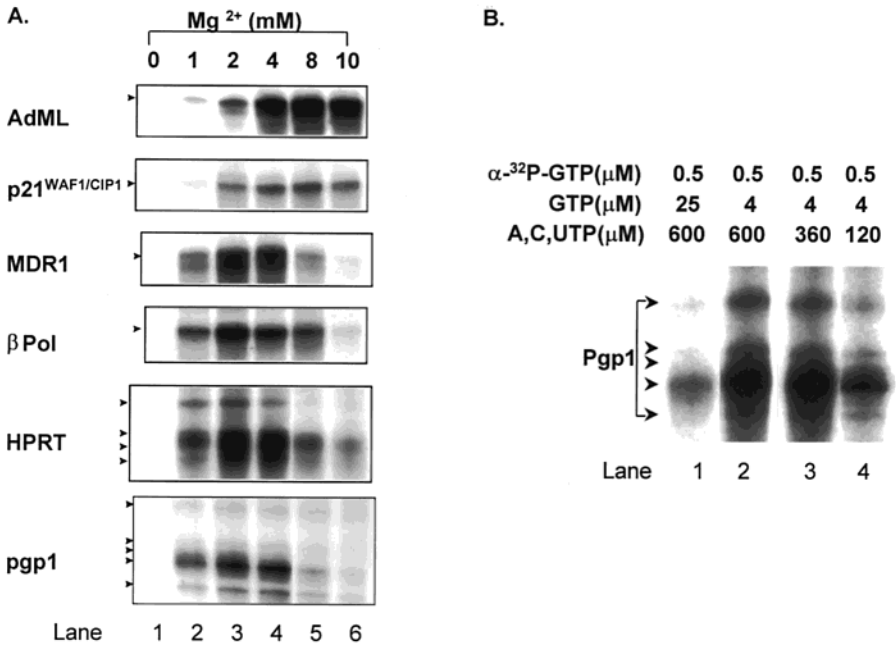

FIGURE 2: Mg^{2+} and nucleotide concentrations are critical for the optimal expression of MSS promoters. (A) In vitro transcription of TATA-containing promoters (AdML, $p21^{WAF1/CIP1}$), TATA-less SSS promoters (MDR1, β Pol), and TATA-less MSS promoters (HPRT, pgp1) requires different optimal Mg^{2+} concentrations. Titration of Mg^{2+} concentrations was performed in semi-MPC conditions as described in Materials and Methods. Abbreviations: β Pol, DNA polymerase β gene; HPRT, hypoxanthine phosphoribosyltransferase gene; MDR1, human multidrug resistance-1 gene. (B) Influence of nucleotide concentration on transcription of the pgp1 promoter. Nucleotide concentrations were titrated under semi-MPC conditions as described in Materials and Methods. Arrows designate transcription start sites.

Short Linear Templates Are Optimal for MSS Transcription. In our early analyses using supercoiled or linearized plasmid templates (5−7 kb), specific transcription from the pgp1 template could not be detected above the background of nonspecific transcription arising from cryptic sites within the plasmid backbone (see Figure 1B, lanes 3 and 7, for linearized plasmid templates). We therefore determined whether the use of shorter linear templates lacking these cryptic start sites would reduce background transcription in favor of accurately initiated transcripts. When a linear ~430 bp PCR fragment was substituted for the full-length plasmid template, nonspecific transcription was markedly reduced, allowing for the visualization of multiple weak but specific pgp1 transcripts (compare lanes 7 and 8 of Figure 1B). To verify that the transcripts observed were initiating at the correct sites, the experiment was repeated, using a longer template in which the 3′ end was extended and the 5′ end was kept constant (Figure 1C, template B). As expected, all five pgp1 transcripts generated from this template were of a higher molecular weight, with the increase in size reflecting elongation through the additional 3′ sequence (Figure 1C, compare lanes 1 and 2). A higher resolution gel showing specific pgp1 transcripts is shown in Figure 1D. Linear templates (~400−500 bp in length) and a nuclear runoff assay with crude nuclear extract were therefore employed for all the promoters analyzed in subsequent optimization experiments.

TATA-Containing, TATA-less SSS, and TATA-less MSS Promoters Require Different Optimal Magnesium Concentrations. Given the importance of both the type and concentration of cations that had been demonstrated for single start site promoters (*25*), we determined the optimal cation concentration for transcription of two MSS promoters, pgp1 and human hypoxanthine phosphoribosyltransferase (HPRT), relative to two TATA-containing SSS promoters (AdML and $p21^{WAF1/CIP1}$) and two TATA-less SSS promoters [the human class I P-glycoprotein homologue multidrug resistance (MDR1) and the DNA polymerase β (β Pol) promoters]. As shown in Figure 2A, optimal transcription from both TATA-less SSS and MSS promoters occurred at low Mg^{2+} concentrations (2 mM), while the transcription of TATA-containing AdML and $p21^{WAF1/CIP1}$ promoters was efficient over a range of Mg^{2+} concentrations, with optimal activity at ~8 mM. Other divalent cations such as Mn^{2+} and Co^{2+} were weak substitutes for Mg^{2+} (data not shown).

Nucleotide Concentration and Specific Activity Influence Transcription of MSS Promoters. To determine the optimal concentration of ribonucleotides in the assay mix, the concentration of the labeled ribonucleotide ([α-^{32}P]GTP) was kept constant (0.5 μM), while the concentration of unlabeled ribonucleotides was varied (Figure 2B). Under standard conditions (25.5 μM [α-^{32}P]GTP and 600 μM unlabeled ATP, UTP, and CTP), transcription from the pgp1 promoter was weak, although all five start sites were detectable (lane 1). Increasing the specific activity of GTP (by decreasing the concentration of unlabeled nucleotide) markedly increased the detection of MSS transcripts (lane 2). Transcription was not noticeably affected when the total nucleotide concentration was reduced by almost half (360 μM, lane 3) but was significantly reduced when the concentration was reduced by 80% (120 μM, lane 4).

Other Parameters Influencing in Vitro Transcription of MSS Promoters. A number of other factors affecting MSS transcription were tested (Figure 3), including template and salt concentration, as well as preincubation and reaction time. Template concentration was found to be linear from 0.2 to 0.9 pmol (panel A). As shown in panel B, the AdML promoter was efficiently transcribed under a fairly broad range of KCl concentrations (~70−110 mM) as previously reported, with an optimal concentration of 100 mM. In

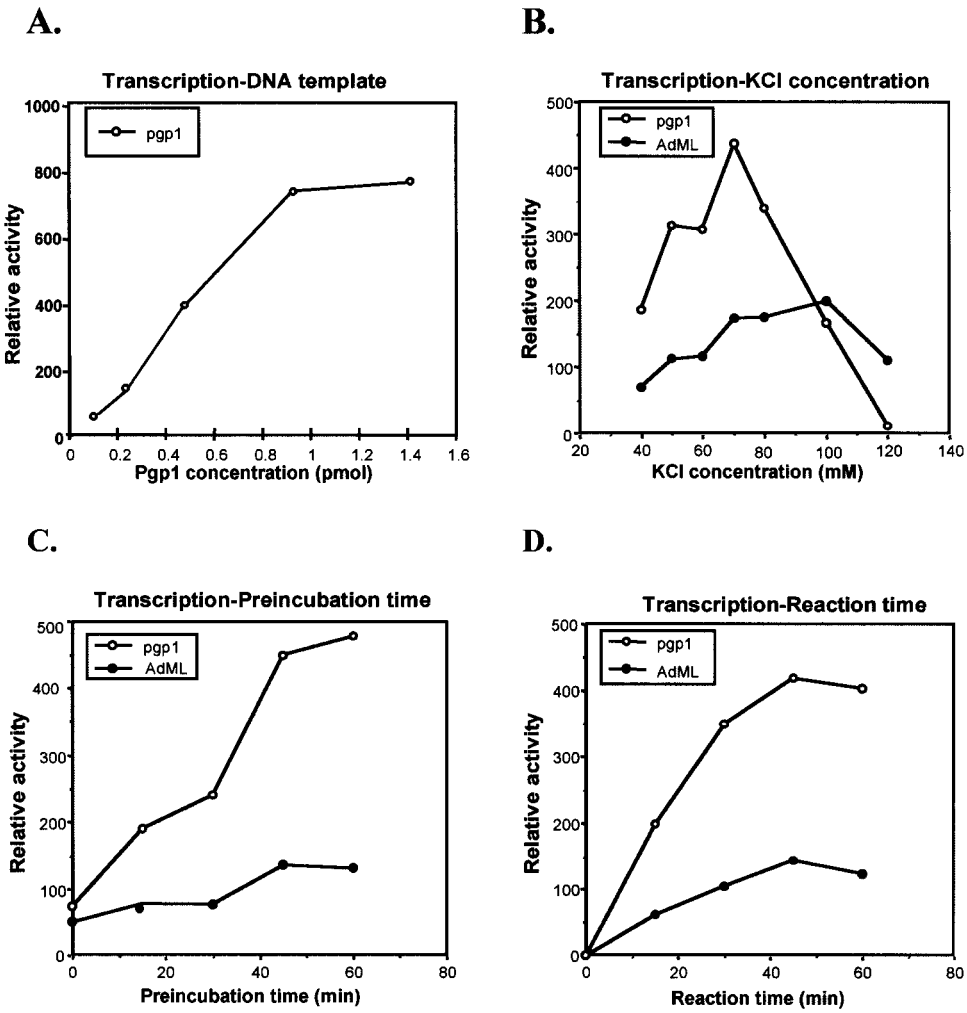

FIGURE 3: Effect of other parameters on transcription of MSS promoters. All assays were performed under semi-MPC conditions where only the parameters to be optimized were varied. Quantitation of the in vitro transcripts was accomplished using a BetaScope RadioImager (Betagen, MA). 0.53 pmol of pgp1 and 0.062 pmol of AdML templates were used in (B), (C), and (D). (A) Effect of template concentration. (B) Effect of KCl concentration. (C) Effect of preincubation time on formation of the PIC. (D) Effect of incubation time on productive transcription initiated by the addition of NTPs.

contrast, transcription from the pgp1 promoter was much more sensitive to salt concentration, with a sharp peak of transcription observed at 70 mM KCl and a rapid decline at higher concentrations (panel B). This was also observed when NaCl was substituted for KCl (data not shown). As has been observed with other TATA-less promoters, formation of the preinitiation complex required preincubation at 27 °C for 45−60 min (panel C); following the addition of nucleotides to the preincubation mix, optimal transcription was achieved in 45 min (panel D). The DTT concentration (1−8 mM) had little effect on transcription; the addition of 2% PEG-8000 and 1% DMSO increased the transcript yield (data not shown).

Effect of the RNA Extraction Method on Nonspecific Background. Under standard conditions, transcription is stopped by the addition of a solution containing salt and sodium dodecyl sulfate; then RNA is extracted by phenol/chloroform and alcohol-precipitated in the presence of tRNA or glycogen. While this is a generally effective method for isolating nucleic acids, its disadvantages include the prolonged handling of isotope during the extraction/precipitation steps and the need to transfer the RNA solution multiple times. We chose instead to isolate RNA by the single step/single tube guanidinium method (*26*), using a pellet paint coprecipitant (Novagen) along with minimal amounts of tRNA as carrier. Isolation of in vitro transcribed pgp1 RNA was equally efficient using the conventional method or the simplified guanidinium method, but we routinely observed a reduction in background bands using the guanidinium method (data not shown). Therefore, this method was adopted for the purification of RNA transcribed in the MPC system.

The MPC Assay System Supports Transcription from All Three RNA Pol II Promoter Classes. Although the MPC assay was developed primarily for the analysis of MSS promoters, its value would increase if it could be used for the analysis of all RNA Pol II promoter classes. We therefore compared transcription of TATA-containing promoters [AdML, $p21^{WAF1/CIP1}$, simian virus 40 (SV40)], TATA-less SSS promoters (β Pol, MDR1), and TATA-less MSS promoters (pgp1, HPRT) under standard and MPC conditions (Figure 4). While transcription of both MSS promoters was barely detectable in the standard mix (lanes 11 and 13), it was readily detected in the MPC assay (lanes 12 and 14). In contrast, transcription of the TATA-containing promoters was not improved in this system (lanes 2, 4, and 6). In fact, transcription of AdML was markedly reduced when MPC conditions were employed (lane 2 vs lane 1), suggesting a fundamental difference in the requirements for transcription

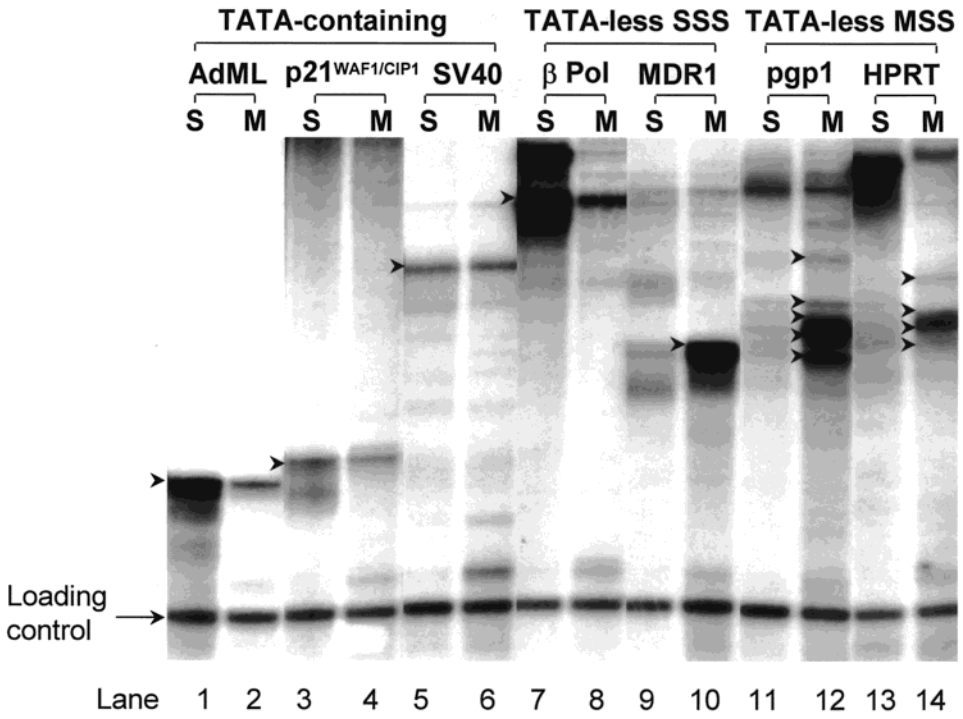

FIGURE 4: Comparison of the in vitro transcription of all three classes of Pol II promoters under MPC versus standard conditions. TATA-containing promoters (AdML, p21$^{WAF1/CIP1}$, SV40), TATA-less SSS promoters (β Pol, MDR1), and TATA-less MSS promoters (pgp1, HPRT) were analyzed under standard (S) and MPC (M) conditions. Template molar concentrations were identical for each promoter under both assay conditions. Arrowheads designate transcription start sites. A radiolabeled PCR product was added to the completed reactions as a loading control.

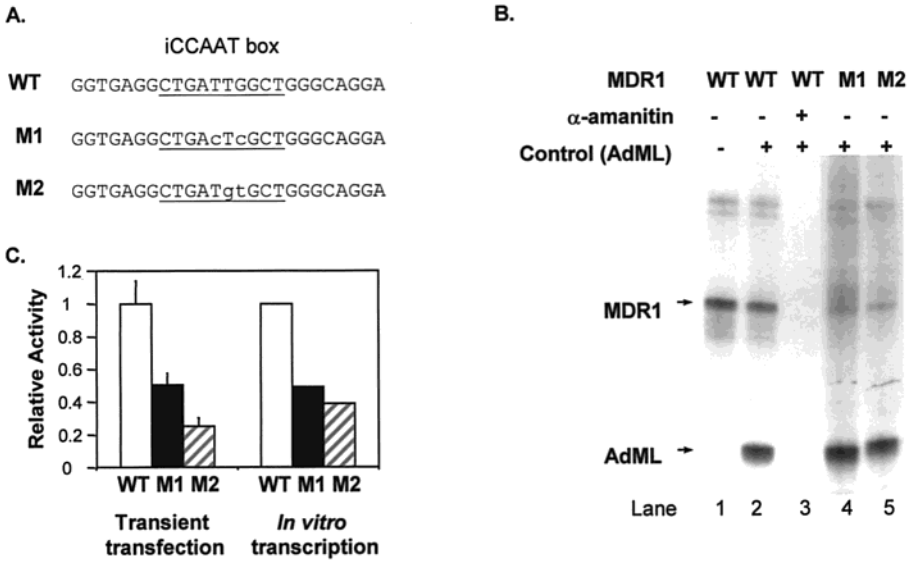

FIGURE 5: In vitro transcription under MPC conditions can be regulated by promoter elements. (A) Sequence of wild-type (WT) and inverted CCAAT (iCCAAT) box mutants of the MDR1 promoter. (B) In vitro transcriptions were performed under MPC conditions. Lanes: 1−3, the WT MDR1 promoter; 4 and 5, two different MDR1 iCCAAT box mutants, M1 and M2. AdML was included in the transcription as an internal control (lanes 2−5). The transcription of MDR1 is α-amanitin sensitive (lane 3). Arrows designate transcription start sites of the MDR1 and AdML promoters. (C) Comparison of the activities of MDR1 WT, M1, and M2 promoter constructs in vivo and in vitro. Left: relative luciferase activities of transiently transfected MDR1 constructs in KB-3-1 cells. Right: quantitation of the in vitro generated MDR1 transcripts shown in (B) normalized against AdML.

of TATA-containing and TATA-less MSS promoters. The assay conditions differentially affected transcription of TATA-less SSS promoters: MDR1 was more efficiently transcribed in the MPC mix (lanes 9 and 10) while β Pol was less efficiently transcribed (lanes 7 and 8). Notably, there was a general decrease in nonspecific transcription from TATA-less promoters under MPC versus standard assay conditions (in particular, compare lanes 7 and 8 and lanes 13 and 14), remarkable considering that all transcripts synthesized in either direction are visualized by this runoff assay.

MPC System Application: In Vitro Transcription of the MDR1 Promoter. Many early in vitro transcription assays developed for the analysis of TATA-containing promoters correctly reproduced basal transcription but failed to recapitulate any of the regulated changes in transcription seen

in vivo. We therefore tested whether the MPC assay was able to recapitulate a known in vivo observation. We have previously shown that mutation of an inverted CCAAT (iCCAAT) box in the MDR1 promoter decreases transcription in vivo (*27*). To examine the role of this element in vitro, linear templates were prepared from the wild-type MDR1 plasmid and from two mutant plasmids, each of which contained a distinct mutation within the iCCAAT element (Figure 5A) (*27*, *28*). As shown in Figure 5B, both mutations resulted in a significant decrease in transcription from the MDR1 promoter template (lanes 4 and 5) when compared to the wild-type template (lanes 1 and 2). This is in agreement with the in vivo data (Figure 5C) and indicates that MPC conditions may be suitable for the analysis of activated as well as basal transcription. Moreover, unlike what has been shown for the effect of template topology on the in vitro regulation of a subset of TATA-containing promoters (*29*, *30*), these results indicate that the linear MDR1 template can be regulated in a physiologically relevant manner under MPC conditions.

DISCUSSION

Despite extensive analysis of TATA-containing and TATA-less SSS RNA Pol II promoters, there has been little effort to address the regulation of TATA-less MSS promoters. The utilization of MSS in vivo can be influenced by a number of biological processes. In the case of the pgp1 promoter, regulation is imposed following selection for resistance to cytotoxic agents (*17*); in the case of the HMGCoA reductase gene, start site utilization is regulated in a tissue-specific manner (*31*). Identifying the transcription factors, chromatin modifiers, and signaling events mediating this regulation will require a significant effort. As an initial step toward dissecting these complex events, we have developed an in vitro system, the MPC assay, which is optimized for the analysis of MSS promoters. Using this system, we have demonstrated accurate transcription of two MSS promoters (the pgp1 promoter and the HPRT promoter), neither of which were transcribed under standard conditions. Moreover, the MPC assay also supported transcription from TATA-containing and TATA-less SSS promoters, making it the first assay system in which all three Pol II promoter classes can be analyzed.

Several parameters were examined during the optimization of the MPC assay. The use of a linear 400−500 bp promoter-containing DNA template instead of a larger (5−7 kb) promoter-containing plasmid template (supercoiled or linear) increased the specific signal. In addition, the background resulting from nonspecific initiation at cryptic regions within the plasmid backbone was eliminated. Not surprisingly, the difference in template length had a more dramatic effect on the detection of weak TATA-less promoters (SSS and MSS), where the signal-to-background ratio can be very low. Unlike TATA-containing promoters, the transcription of MSS promoters was restricted within a narrow window of Mg^{2+} concentration, with an optimal concentration of 2 mM. Efficient transcription of the MSS promoters was observed at a lower concentration of ribonucleotides (and higher specific activity of labeled nucleotide) than is commonly employed under standard conditions. Interestingly, optimization of the MPC assay for MSS promoters did not result in increased expression of TATA-containing promoters. In fact, the TATA-containing AdML and the TATA-less SSS β Pol promoter were expressed much less efficiently in the MPC assay than in the standard assay. It is not known whether the absence of a TATA box and the apparently non-DNA-binding role of TBP is the only difference between transcription initiation in TATA-containing and TATA-less promoters. The narrow window of salt and cation requirements for MSS promoters versus TATA-containing promoters may point to a more fundamental difference in how transcription is achieved in this class of RNA Pol II promoters. That multiple promoters in each class were tested in this study suggests that these differences are not gene specific but class specific.

We have previously shown that MSS utilization within the pgp1 promoter is differentially regulated in vivo; the +1 site is preferentially utilized in parental drug-sensitive Chinese hamster cells while the downstream start sites are also utilized in drug-resistant variants. Notably, this regulation can only be reproduced in transfection assays when the reporter construct is stably integrated, suggesting a role for chromatin in the regulation of start site utilization. The mechanism underlying this regulation remains unclear, but two reasonable hypotheses emerged from the in vivo studies. In one scenario, +1 site utilization is the "default" pathway, while initiation at downstream start sites is a "regulated" event. In the second case, utilization of all five start sites is the default event, and suppression of downstream start sites is regulated. The recapitulation of MSS utilization in our MPC in vitro assay, in which the DNA template is devoid of nucleosomes and thus not subjected to higher order chromatin regulation, supports the second hypothesis. However, it should be noted that, while the relative utilization of the downstream start sites was similar in vitro to what had been observed in vivo, the transcription of +1 was markedly weaker in vitro. This difference was consistently observed, even when assay parameters were varied. While the basis for this discrepancy between the in vivo and in vitro results remains unclear, recent preliminary data suggest that the +1 site is regulated by activators distinct from those that regulate the downstream start sites. It is possible that such "activators" are not functional in our crude HeLa extracts; further analyses of the regulation of +1 in vitro are underway.

To our knowledge, the MPC assay is the first system that allows transcription from all three classes of RNA Pol II promoters, and this is the first study in which seven promoters of all three promoter classes have been examined simultaneously using the same in vitro assay. This provides a tool for studying the transcriptional regulation of promoters previously beyond the scope of the in vitro study and lays the groundwork for a more detailed examination of MSS promoter regulation in vitro, where the roles of individual promoter elements, components of the basal machinery, transcriptional activators, and chromatin structure can be examined.

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